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Effects of High Fat Diet-Induced Obesity on Expression of Vitamin D Metabolizing Enzymes in Mice

고지방 식이로 유도한 비만이 마우스의 비타민 D 대사 관련 효소 발현에 미치는 영향

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Abstract

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Low serum 25-hydroxyvitamin D (25(OH)D) concentration has been often reported in obese human. Lifestyle factors such as inadequate sun exposure and physiological factors such as impaired hepatic 25-hydroxylation have been suggested as possible explanations for vitamin D deficiency in obesity, however, the precise mechanism has not been elucidated. We investigated the effects of obesity on vitamin D status and the expression of vitamin D metabolizing enzymes using high fat diet-induced obese mice in order to understand the underlying mechanisms of altered vitamin D metabolism associated with obesity. Four-wk-old C57BL/6 mice were fed control diet containing 10% energy fat (control group) or high fat diet with 45% energy fat (obese group) for 18 weeks. There was no difference in serum 25(OH)D

concentration between groups while serum 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) concentration was significantly higher in obese mice. Hepatic mRNA levels of 25-hydroxylases (*Cyp2r1*, *Cyp27a1* and *Cyp2j3*) were lower in obese group, but CYP27A1 protein expression did not differ between groups. Renal 1α-hydroxylase (*Cyp27b1*) mRNA level was upregulated and 24-hydroxylase (*Cyp24*) mRNA level was downregulated in obese group. Serum $1,25(OH)_2D$ concentration correlated positively with renal *Cyp27b1* expression level and negatively with renal *Cyp24* expression level. No difference in serum calcium level was observed between groups, but serum parathyroid hormone (PTH) concentration was higher in obese mice. Serum concentration of PTH correlated positively with body weight. In visceral adipose tissue, mRNA levels of 25-hydroxylases, *Cyp27a1* and *Cyp2j3*, and vitamin D receptor (*Vdr*) were higher in obese mice. Overall, the expression of vitamin D metabolizing enzymes was influenced by high fat diet-induced obesity, which might partly explain the mechanisms of altered vitamin D endocrine system in obesity. Higher concentration of serum PTH and $1,25(OH)_2D$ in obese mice suggests abnormal regulation of serum $1,25(OH)_2D$ concentrations by secondary hyperparathyroidism, which might have contributed to lower hepatic 25-hydroxylase mRNA levels. Higher mRNA expression of *Vdr* in adipose tissue might suggest the possibility of

enhanced nonclassic functions by the interaction between 1,25$(OH)_2$D and VDR in adipose tissue.

KEY WORDS: Obesity, 25-hydroxyvitamin D, 1,25-dihydroxyvitamin D, Hepatic 25-hydroxylase, Renal 1α-hydroxylase, Renal 24-hydroxylase, High fat diet-fed mice

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List of Abbreviations

11β-HSD, 11β-hydroxysteroid dehydrogenase

1,25$(OH)_2$D, 1,25-dihydroxyvitamin D

1,25D_3 MARRS, 1,25D_3 membrane-associated rapid response to steroid

25(OH)D, 25-hydroxyvitamin D

Ca, calcium

DBP, vitamin D binding protein

Gapdh, glyceraldehyde 3-phosphate dehydrogenase

nVDR, nuclear vitamin D receptor

PTH, parathyroid hormone

RIA, radioimmunoassay

RXR, retinoid X receptor

UCP, uncoupling protein

VAT, visceral adipose tissue

VDR, vitamin D receptor

VDRE, vitamin D receptor element

I. Introduction

It has been observed that obese subjects are more likely to have vitamin D deficiency (serum 25(OH)D concentration below 20 ng/mL) or insufficiency (serum 25(OH)D concentration between 21 and 29 ng/mL) (Earthman et al., 2012). An inverse relationship between body mass index (BMI) and serum 25(OH)D has been reported in obese human (Cheng et al., 2010, Drincic et al., 2012, Looker, 2005). In addition, an increase in serum 25(OH)D after significant weight loss following bariatric surgery was observed in obese patients who had low preoperative levels of serum 25(OH)D (Compher et al., 2008).

Various explanations suggested for low 25(OH)D status associated with obesity were limited exposure to the sun light due to decreased activity and sequestration of vitamin D_3 in adipose tissue (Earthman et al., 2012). Recently, challenging the sequestration hypothesis, simple volumetric dilutional model was suggested as another explanation (Drincic et al., 2012). An impaired hepatic synthesis of 25(OH)D attributed to elevated levels of $1,25(OH)_2D$ and PTH was also suggested as a reason for low 25(OH)D status in obesity (Bell et al., 1985, Bell et al., 1984). Obese human was shown to have higher PTH and $1,25(OH)_2D$ levels and lower 25(OH)D level (Bell et al., 1985). And, $1,25(OH)_2D$ was suggested to inhibit hepatic synthesis of

25(OH)D as administration of 1,25$(OH)_2$D in normal subjects prevented the increase in serum 25(OH)D in response to 2.5 mg of vitamin D challenge (Bell et al., 1984). However, the precise mechanism for low 25(OH)D status associated with obesity is still not clear.

Vitamin D metabolism mainly consists of hydroxylations at three different positions (1, 25, and 24) by hydroxylases that function coordinately to metabolize vitamin D and its metabolites. Once vitamin D is formed in skin via UV-light dependent reaction or obtained from the diet, it is transported in circulation to the liver where it is converted to 25(OH)D by 25-hydroxylases including CYP2R1, CYP27A1, and CYP2J3. The major circulating form of vitamin D, 25(OH)D, is further hydroxylated by 1α-hydroxylase (CYP27B1) in the kidney to the hormonally active 1,25$(OH)_2$D. 25(OH)D and 1,25$(OH)_2$D can be catabolized by 24-hydroxylase (CYP24) (Jones et al., 2012). 1,25$(OH)_2$D exerts its classical functions related to bone mineral homeostasis by binding to the vitamin D receptor (VDR) in the gut and kidney, but, expression of VDR has been also reported in other tissues including adipose tissue which suggests the diverse effects of vitamin D beyond its classical roles in bone metabolism (Bikle, 2009). In addition, the expression of vitamin D metabolizing enzymes was observed and the presence of local vitamin D metabolism was suggested in human adipose tissue (Wamberg et al., 2013).

Under normal physiological conditions, the level of serum 1,25$(OH)_2$D is tightly controlled by the regulation of renal 1α-hydroxylase and 24-hydroxylase activities. PTH and 1,25$(OH)_2$D itself are considered as the most important regulators for the expression of these two enzymes (Omdahl et al., 2002). However, increased serum 1,25$(OH)_2$D level along with high level of serum PTH has been reported in obese human (Bell et al., 1985, Breslau, 1988). On the other hand, an inverse association between serum 1,25$(OH)_2$D level and BMI was observed in other studies (Konradsen et al., 2008, Parikh et al., 2004). These evidences suggest altered vitamin D metabolism associated with obesity, however, precise mechanisms have not been elucidated.

Mice have been successfully used as animal models to investigate the mechanism of vitamin D. Effects of high dose of vitamin D on calcium and vitamin D metabolism (Fleet et al., 2008) and the impact of long-term dietary vitamin D deficiency on brain functions (Brouwer-Brolsma et al., 2014) have been examined using mice models, both of which were not feasible in human studies due to difficulties in tissue collection and lifestyle modification on diet.

In this study, we used high fat diet-induced obese mice model to minimize the differences in lifestyle related factors in obese human such as sun exposure and dietary vitamin D intake. We investigated the vitamin D status

and the expression of enzymes involved in vitamin D metabolism in order to elucidate the mechanisms responsible for obesity related changes in vitamin D status. The specific objectives for this study were to investigate 1) whether circulating levels of major vitamin D metabolites were influenced by obesity; 2) whether there were any changes in the expression levels of the vitamin D metabolizing enzymes in liver and kidney due to obesity; 3) whether expression levels of these metabolizing enzymes correlated with the serum levels of vitamin D metabolites; and 4) whether expressions of vitamin D metabolizing enzymes and VDR in adipose tissue were affected by obesity.

II. Literature Review

1. Obesity and vitamin D

Obesity has been recognized as a global health problem of great importance and related to many different comorbidities such as dyslipidemia, insulin resistance and cardiovascular disease. Interestingly, vitamin D deficiency or inadequacy are also highlighted as the major health problem with evidence for links to these chronic diseases. It has long been reported that obese subjects frequently have vitamin D deficiency or insufficiency (serum 25(OH)D concentration below 30 ng/mL), but underlying mechanism between these two major health problems has not been elucidated yet (Holick, 2007).

As a contributor to low 25(OH)D status in obesity, various explanations have been suggested, such as: less skin exposure to the sun, impaired ability of hepatic synthesis of 25(OH)D by elevated levels of $1,25(OH)_2D$ and PTH, and sequestration of vitamin D_3 in adipose tissue which has been most often invoked (Earthman et al., 2012). Recently, simple volumetric dilutional model of vitamin D_3 between serum and adipose tissue was suggested as another explanation for the low 25(OH)D status in obesity (Drincic et al., 2012).

The relationship between obesity and serum level of the active form of vitamin D, 1,25$(OH)_2$D, is not settled yet. The concentration of serum 1,25$(OH)_2$D is tightly controlled by regulating activities of renal 1α-hydroxylase and 24-hydroxylase under normal physiological conditions. PTH and 1,25$(OH)_2$D itself are considered as the most important regulators of these two enzymes' expression (Omdahl et al., 2002). However, the level of serum 1,25$(OH)_2$D has been reported to increase in obese human (Breslau, 1988), while there are other studies which observed inverse association between serum 1,25$(OH)_2$D level and obesity. They suggested that serum level of 1,25$(OH)_2$D depends on the availability of substrate, 25(OH)D, so low level of serum 1,25$(OH)_2$D is also observed in obesity, influenced by decreased level of serum 25(OH)D (Konradsen et al., 2008).

2. Metabolic pathway of vitamin D

Vitamin D_3 is synthesized in the skin from 7-dehydrocholestrol through exposure to ultraviolet irradiation and it can also be taken from the diet. Vitamin D_3 is transported in the blood by vitamin D-binding protein (DBP) to the liver where it is hydroxylated by hepatic 25-hydroxylases including CYP2R1, CYP27A1, and CYP2J3. The resulting form, $25(OH)D_3$ is the major circulating vitamin D-metabolite which is transported by DBP to the kidney. Further hydroxylation of $25(OH)D_3$ is occurred by 1α-hydroxylase (CYP27B1) to produce $1,25(OH)_2D_3$, the biologically active form of vitamin D (Christakos et al., 2010). Additionally, the 24-hydroxylase (CYP24) is clearly known to exist in normal physiology to catabolize $25(OH)D_3$ and degrade $1,25(OH)_2D_3$ to inhibit its biological activity (Jones et al., 2012). Meanwhile, it should be noted that another major form of vitamin D, vitamin D_2, undergoes similar metabolic transformations as vitamin D_3. Since vitamin D_3 and vitamin D_2 provide various contributions to the overall vitamin D status of the body, it is important that both forms are measured totally (Henry, 2011). Therefore, references to vitamin D or its metabolites below will refer to both forms unless otherwise indicated with a specific subscript.

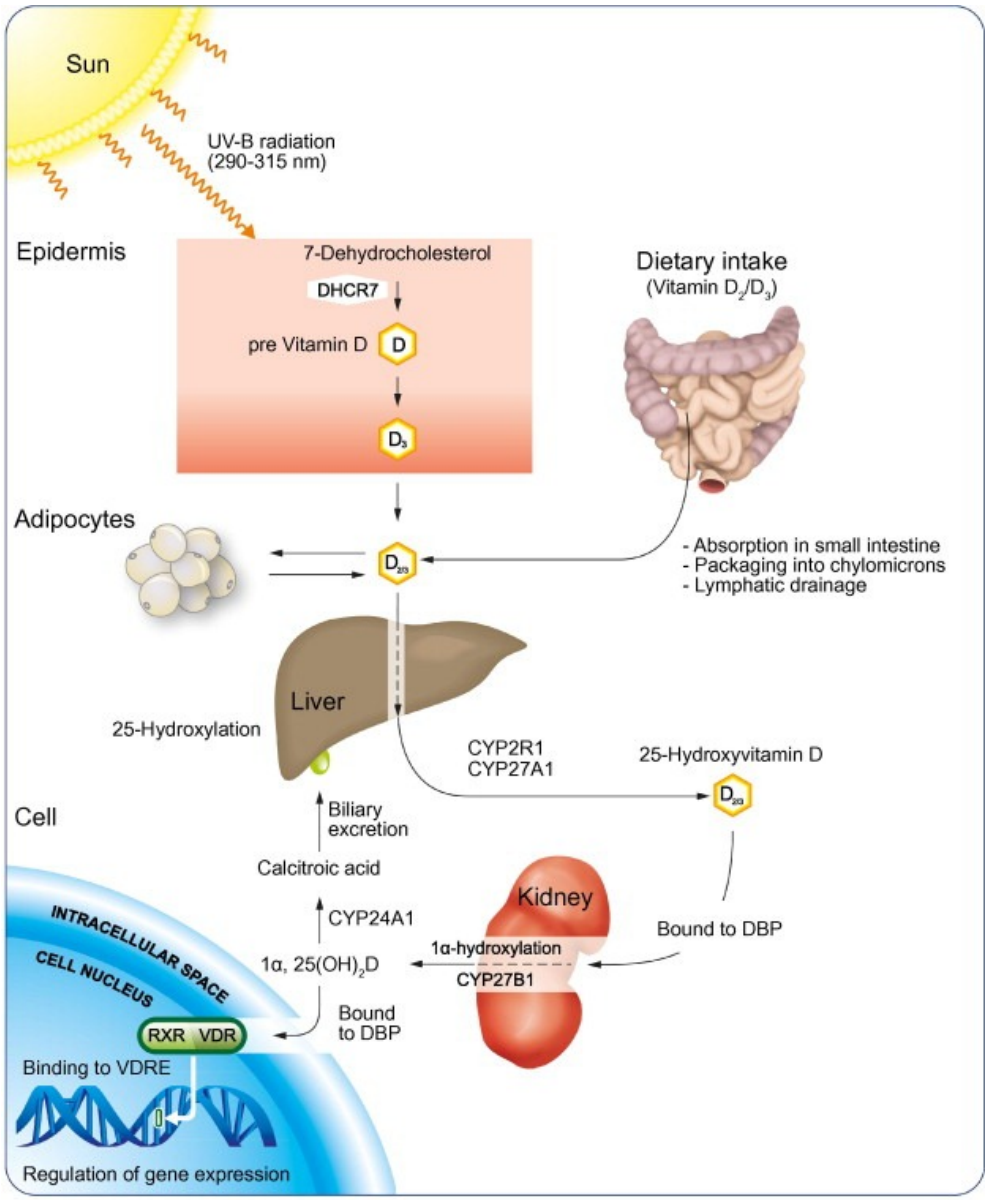

Figure 1. The metabolic pathway of vitamin D[1] (Kitson et al., 2012)

[1]CYP2R1, CYP27A1: 25-hydroxylase; CYP24A1: 24-hydroxylase; CYP27B1: 1α-hydroxylase; DBP, vitamin D binding protein; RXR, retinoid X receptor; VDR, vitamin D receptor; VDRE, vitamin D receptor element.

Vitamin D hydroxylases have a P450 notation by nomenclature rules derived for cytochrome P450 superfamily because they were found to contain heme-binding and other functional domains typical of cytochrome P450 hemoprotein enzymes (Henry, 2011). Cytochrome P450-dependent hydroxylases are integral membrane proteins located in either the mitochondria or smooth endoplasmic reticulum and require electrons from NADPH to reduce molecular O_2 to one hydroxyl group and one water molecule. The terms P450C25, P450C1, and P450C24 are used to describe these three hydroxylases, and they are also named using the term CYP which represents cytochrome P450 (Omdahl et al., 2002).

25-hydroxylase

Since the liver was identified as the major site of 25-hydroxylation of vitamin D, there have been several candidates for the 25-hydroxylases. CYP2R1 was found to be a highly-conserved and substrate-specific microsomal 25-hydroxylase. CYP27A1 was reported to additionally display 25-hydroxylation of vitamin D and referred to as mitochondrial 25-hydroxylase.

Recently, other microsomal 25-hydroxylases were also identified such as CYP2J2/3, CYP3A4, CYP2D11, and CYP2D25, while only CYP2J2/3 and CYP3A4 seem to match the enzymatic properties (Prosser et al., 2004). CYP2J3 has been reported to exist only in rat with comparable expression

level to that of CYP27A1, and CYP2J2 might be a possible equivalent candidate in human but its true physiological meaning in vitamin D bioactivation needs further investigation. CYP3A4 is more likely the 24/25-hydroxylase responsible for the metabolism of vitamin D prodrugs and other related therapeutics.

Among these possible 25-hydroxylases, CYP2R1 is considered as the physiological vitamin D 25-hydroxylase at least in human, but is less abundant than CYP27A1 in the liver of mouse and rat (Zhu et al., 2012). Also, the mitochondrial vitamin D 25-hydroxylase, CYP27A1, was reported as the major 25-hydroxylase in female rat liver, showing 150% higher affinity than microsomal 25-hydroxylases (Masumoto et al., 1988).

1α-hydroxylase

The second step of activation, 1α-hydroxylation of 25(OH)D, mainly occurs in the proximal renal tubule by 1α-hydroxylase (CYP27B1). The bioactivation of 25(OH)D to 1,25$(OH)_2$D proceeds at variable rates due to regulation of the enzyme's expression level and the affinity of this enzyme differs between species with wide range of Km values. Apart from the major substrate, 25(OH)D, 24,25$(OH)_2$D is also the preferred substrate for CYP27B1. However, the rate of 1,25$(OH)_2$D synthesis is greater than that of

1,24,25$(OH)_3$D synthesis because of the 10-fold higher concentration of 25(OH)D (Omdahl et al., 2002).

It is now known that CYP27B1 is also found in extrarenal sites including placenta, macrophage, keratinocytes, breast, adipose tissue, and so on. Meanwhile, the extrarenal CYP27B1 activity is not regulated by the classical calcium and phosphate-regulating hormones, but depends specifically on the cell's environment or functions. The extrarenal existence and activity of CYP27B1 imply that locally-produced 1,25$(OH)_2$D may increase in various intracellular sites by this enzyme and locally trigger nonclassic functions. However, further studies are needed to test the specific role of CYP27B1 in extrarenal sites (Adams et al., 2012).

24-hydroxylase

24-hydroxylase (CYP24) is a multicatalytic enzyme that mainly catalyzes two substrates, 25(OH)D and 1,25$(OH)_2$D, the latter with a 10-fold higher efficiency. Almost all cells express CYP24 *in vivo*, but the highest activity is observed in kidney (Omdahl et al., 2002). CYP24 accelerates the catabolism of 1,25$(OH)_2$D to 1,24,25$(OH)_3$D and produces 24,25$(OH)_2$D from 25(OH)D which is available for 1α-hydroxylation, resulting in decreasing the amount of 1,25$(OH)_2$D in target tissues (Christakos et al., 2010). Thus, CYP24 plays an important role in the inactivation and clearance of excess

vitamin D, controlling 1,25$(OH)_2$D levels. Meanwhile, there is evidence that one of the resultants of CYP24, 24,25$(OH)_2$D, has biological properties distinct from 1,25$(OH)_2$D, but additional evidences are required (Henry, 2011).

3. Regulation of the serum 1,25-dihydroxyvitamin D

Regulation of the vitamin D metabolism mainly focuses on controlling serum $1,25(OH)_2D$ level. The serum concentration of $1,25(OH)_2D$ can be modulated by a series of negative and positive feedbacks, resulting in changes in the expression of the hydroxylases depending on the physiological state (Prosser et al., 2004). Low dietary intake of calcium and phosphate enhances CYP27B1 activity and the elevated level of PTH stimulates transcription of CYP27B1. The resultant, $1,25(OH)_2D$ itself, can negatively regulate CYP27B1. The inactivating hydroxylase of vitamin D, CYP24, is reciprocally regulated when compared to CYP27B1 regulation. It is stimulated by $1,25(OH)_2D$ and inhibited by low serum concentrations of calcium and PTH (Christakos et al., 2010).

Meanwhile, there are some evidences that 25-hydroxylation of vitamin D is regulated by circulating $1,25(OH)_2D$, so $1,25(OH)_2D$ also acts to limit production of its precursor, 25(OH)D (Bell et al., 1984). The increased serum $1,25(OH)_2D$ may reduce hepatic synthesis of 25(OH)D by feedback inhibition (Bell et al., 1985).

4. Vitamin D receptor

The vitamin D receptor (VDR) is a member of the nuclear receptor superfamily which mediates the actions of $1,25(OH)_2D$, the hormonal form of vitamin D. VDR binds to $1,25(OH)_2D$ with high affinity and specificity, then heterodimerises with a retinoid X receptor (RXR) (Ding et al., 2012).

Once the liganded VDR-RXR heterodimer recognizes vitamin D responsive elements (VDREs) in the DNA sequence of vitamin D-regulated genes, a genomic response is generated; the formation of large complexes that can facilitate the expression of the targeted gene (coactivators) or inhibit its expression (cosuppressors). Different tissues have varying levels of these coregulators, providing some degree of tissue specificity for vitamin D action (Bikle, 2010).

VDR is clearly present in cells of the intestinal epithelium, renal tubules, parathyroid gland cells, skin (keratinocytes), mammary epithelium, pancreas (beta islet cells), pituitary gland, skeleton (osteoblasts and chondrocytes), immune system (monocytes, macrophages, and T-lymphocytes), and germ tissues (Wang et al., 2012). The wide-spread existence of VDR may imply the various effects of vitamin D beyond its classical role in calcium homeostasis and bone metabolism. Data from clinical studies have provided a mechanistic basis for the link between vitamin D deficiency or insufficien-

cy and lots of disorders, such as inflammatory bowel disease, diabetes, the metabolic syndrome, and certain types of cancer. However, the specific functions of VDR in many other tissues and cells still remain to be determined (Ding et al., 2012).

5. Nonclassic functions of 1,25-dihydroxyvitamin D in adipose tissue

$1,25(OH)_2D$ has long been recognized to involve in the regulation of calcium and phosphate metabolism mediated by VDR. As $1,25(OH)_2D$ exerts its functions by binding VDR, the tissues participating in bone mineral homeostasis such as gut and kidney have been considered as major tissues retaining VDR (Bikle, 2009).

However, VDR has been found in other various tissues, and the $1,25(OH)_2D$-VDR interaction in these tissues regulates transcription of a number of genes involved in the regulation of many different nonclassic functions such as cell proliferation and differentiation, immune function and metabolism (Wamberg et al., 2013).

VDR has also been reported to exist in either human or mouse adipose tissue. It implies that adipose tissue can be a target tissue for $1,25(OH)_2D$ and have nonclassic functions in it. There are some *in vitro* studies reporting that $1,25(OH)_2D$ may inhibit adipogenesis by suppressing the expression of the key adipogenic transcription factors and reducing lipid accumulation in adipocytes (Kong et al., 2006).

On the other hand, recent functional studies of VDR *in vivo* produced opposite results; VDR-null mice were resistant to high fat diet-induced obesity

probably owing to increases in energy expenditure and fatty acid β-oxidation (Wong et al., 2009), and targeted expression of VDR in adipocytes induced obesity in mice mainly due to reduced energy expenditure with decreased fatty acid β-oxidation and lipolysis in the adipose tissue (Wong et al., 2011). In addition, another *in vitro* study suggested that 1,25$(OH)_2$D-VDR interaction exerts an inhibitory effect on adipocyte UCP2 expression, modulating adipocyte lipid metabolism and energy homeostasis and local functions (Zemel et al., 2008). Taken together, the current data are inconclusive and additional experimental and translational studies are needed to unravel the role of 1,25$(OH)_2$D and VDR in adipose tissue (Ding et al., 2012).

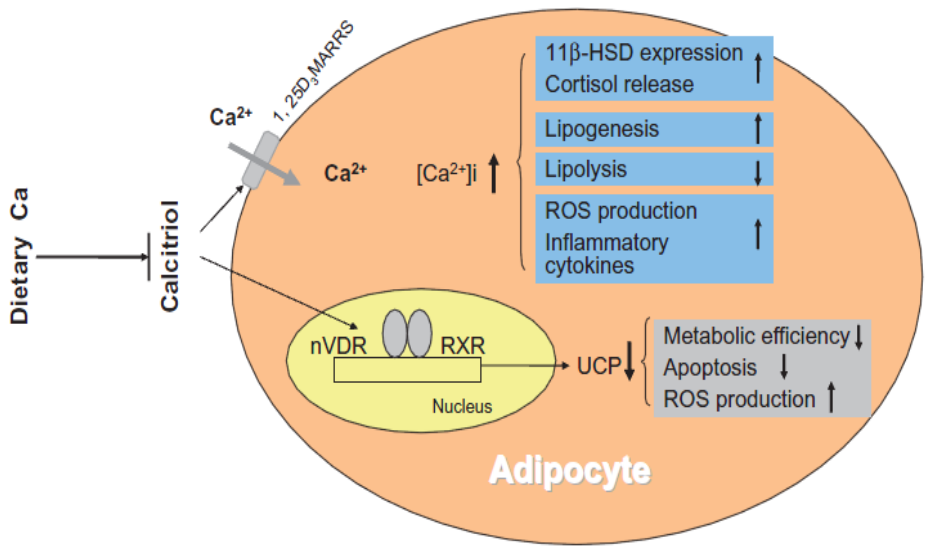

Figure 2. The potential functions of $1,25(OH)_2D$ in adipose tissue[1] (Zemel et al., 2008).

[1] $1,25D_3$ MARRS, $1,25D_3$ membrane-associated rapid response to steroid; 11β-HSD, 11β-hydroxysteroid dehydrogenase; Calcitriol, 1,25-dihydroxyvitamin D ($1,25(OH)_2D$); RXR, retinoid X receptor; UCP, uncoupling protein; VDR, vitamin D receptor

III. Materials and Methods

1. Animals and diets

Four-wk-old male C57BL/6 mice were purchased from Central Laboratory Animal Inc. and individually housed under controlled temperature (23 ± 3°C), humidity (55 ± 10%), and light/dark cycle (12h/12h) conditions in a specific pathogen-free room. Mice were fed a control diet during three days of acclimation period, then divided into control diet group (control group; 10% energy as fat, n = 14) or high fat diet group (obese group; 45% energy as fat, n = 14). Feeding high fat diet containing 45% kcal as fat for 51 days was reported to induce obesity in C57BL/6 mice (Van Heek et al., 1997). The composition of experimental diets (Dyets Inc.) is shown in **Table 1**.

Diets were fed *ad libitum* for 18 weeks and dietary intake was assessed every other day. Animals were weighed once a week. At the end of the experimental period, mice were euthanized by CO_2 asphyxiation after 12 hours of fasting. Blood was obtained by cardiac puncture, serum was isolated by centrifugation at 3,000 rpm for 20 minutes after coagulation, and stored at −80°C. Liver, kidney, and white adipose tissue (epididymal, subcutaneous, perirenal-retroperitoneal, and visceral fat depots) were removed and weighed. All tissues were frozen in liquid nitrogen and stored at −80°C until analysis. All animal protocols were approved by Seoul National Universi-

ty Institutional Animal Care and Use Committee (approval no. SNU-120709-1).

Table 1. Composition of the experimental diets[1]

Item	Control diet		High-fat diet	
	gram	*kcal*	*gram*	*kcal*
Casein	200	716	200	716
L-Cystine	3	12	3	12
Sucrose	350	1400	172.8	691
Cornstarch	315	1134	72.8	262
Dyetrose[2]	35	133	100	380
Soybean Oil	45	405	45	405
Lard	0	0	157.5	1418
t-Butylhydroquinone	0.009	0	0.009	0
Cellulose	50	0	50	0
Mineral Mix[3]	35	16.5	35	16.5
Vitamin Mix[4]	10	39.2	10	39.2
Choline Bitartrate	2	0	2	0
Total	1045.009	3855.7	848.109	3939.7
kcal/gram diet	3.69		4.65	

[1]Resource: Dyets, Inc.

[2]Dyetrose (Dyets) is dextrinized cornstarch containing 90-94% tetrasaccharides.

[3]Thirty-five grams of mineral mix (Dyets, #210099) provides 5.1 g calcium, 4 g phosphorus, 3.6 g potassium, 1 g sodium, 1.6 g chloride, 0.5 g magnesium, 0.3 g sulfur, 59 mg manganese, 46 mg iron, 25 mg zinc, 5 mg copper, 0.2 mg selenium, 0.2 mg iodine, and 4.2 g sucrose.

[4]Ten grams of vitamin mix (Dyets, #300050) provides 4,000 IU vitamin A, 1,000 IU vitamin D_3, 50 IU vitamin E, 30 mg niacin, 16 mg pantothenic acid, 7 mg vitamin B_6, 6 mg vitamin B_1, 6 mg vitamin B_2, 2 mg folic acid, 0.8 mg menadione, 0.2 mg biotin, 10 μg vitamin B_{12} and 9.8 g sucrose.

2. Quantification of serum 25-hydroxyvitamin D

Serum 25(OH)D was measured using a commercial 25-Hydroxyvitamin D ^{125}I RIA kit from DiaSorin (DiaSorin Inc.) as directed by the manufacturers' product inserts. The 25(OH)D analysis largely consists of a two-step procedure. The first procedure is a rapid extraction of 25(OH)D and other hydroxylated metabolites from serum with acetonitrile and the following procedure is assaying the extracted sample using an equilibrium radioimmunoassay (RIA) method based on an antibody with specificity to 25(OH)D.

In the extraction procedure, 500 μL of acetonitirile was added to each 10 x 75 mm borosilicate glass tube for each calibrator, control and serum sample. Six 25(OH)D calibrators at concentrations ranging from 0-100 ng/mL and two 25(OH)D controls (low-normal and high-normal range) were provided from the kit. Fifty μL of calibrators, controls and serum samples were added slowly into the acetonitrile. After vortexing for 10 seconds, all tubes were centrifuged at 1,200 ×g for 10 minutes at 25°C. Twenty-five μL aliquots from the supernatant were transferred in duplicate to each fresh tube.

The whole RIA procedure was performed in the RIA laboratory. In addition to tubes from extraction procedure, Total counts (Tcts) and Non-specific binding (NSB) tubes were also prepared. Whereas nothing was added to Tcts tubes, 25 μL aliquots from the supernatant of extracted Calibrator 0 (0

ng/ml) were transferred in duplicate to each NSB tube. Fifty μL of ^{125}I 25(OH)D tracer and 1 mL of 25(OH)D antiserum were added to each tube of extracted calibrators, controls and serum samples, but 1 mL of NSB buffer was added instead of 25(OH)D antiserum to each Tcts and NSB tube. After vortexing without allowing the contents to foam, all tubes were incubated for 90 minutes at room temperature. Five hundred μL of Donkey Anti-Goat precipitating complex was added to all tubes except for Tcts tubes. All tubes were vortexed gently to mix well and centrifuged at 1,800 ×g for 20 minutes at 25°C. Supernatants were decanted to leave pellets only, then each tube was counted in the Gamma scintillation counter (2480 WIZARD2 ® gamma counter, PerkinElmer) for 1 minute.

3. Quantification of serum 1,25-dihydroxyvitamin D

Serum $1,25(OH)_2D$ was also analyzed via RIA using a commercial 1,25-Dihydroxyvitamin D ^{125}I RIA kit from Immunodiagnostic Systems (IDS Ltd.). The assay system consists of two main steps; the first step is a purification of $1,25(OH)_2D$ in samples and controls by immunoextraction and the following step is quantification of $1,25(OH)_2D$ by ^{125}I RIA.

In the immunoextraction step, two immunocapsules containing monoclonal antibody to $1,25(OH)_2D$ were prepared for each delipidated control and serum sample. A hundred μL of controls and serum samples was added to immunocapsules and all immunocapsules were rotated end-over-end at 10 revolution per minute for 3 hours at room temperature. After removing screw caps and bottom stoppers, all immunocapsules were placed in each labelled plastic tube and centrifuged at 700 ×g for 60 seconds at 4°C. Five hundred μL of diluted Wash Solution was added to each immunocapsule and all tubes with immunocapsules were centrifuged at 700 ×g for 60 seconds at 4°C. After repeating this washing step again, the labelled borosilicate glass tubes, one for each immunocapsule, were prepared and all immunocapsules were transferred to each glass tube. A hundred and fifty μL of Elution Reagent, which elutes $1,25(OH)_2D$ from immunocapsules, was added to each immunocapsule and incubated for 2 minutes. All tubes with

immunocapsules were centrifuged at 700 ×g for 60 seconds at 4°C and this elution step was repeated two times. Immunocapsules were discarded and the elutes in the glass tubes were evaporated under a gentle flow of nitrogen for 20-30 minutes at 30°C to leave white pellet only in the tubes. A hundred μL of Assay Buffer was added to each tube and then the immunopurified samples and controls were ready for the RIA step.

The whole RIA procedure was performed in the RIA laboratory. In addition to extract tubes from immunoextraction step, appropriately labelled borosilicate glass tubes were also prepared two for each calibrator, Non-specific binding (NSB), and Total Counts (Tcts). A hundred μL of each calibrator was added to each labelled tube and 300 μL of Assay Buffer was added to NSB tubes. Except NSB tubes, 200 μL of Primary Antibody (Ab) was added to all tubes. Then all tubes were vortexed gently without foaming and incubated for 16 hours at 4°C. Two hundred μL of ^{125}I 1,25$(OH)_2$D Assay Buffer was added to all tubes including NSB and Tcts tubes. After vortexing without allowing the contents to foam, all tubes were incubated for an hour at room temperature. Four mL of diluted Wash Solution was added to all tubes except Tcts tubes. All tubes were centrifuged at 2,000 ×g for 20 minutes at 4°C. Supernatants were decanted to leave pellets only, then each tube was counted in the Gamma scintillation counter (2480 WIZARD2 ® gamma counter, PerkinElmer) for 1 minute.

4. Quantification of serum parathyroid hormone

Serum PTH was quantified by two-site enzyme-linked immunosorbent assay (ELISA) with the commercial mouse intact parathyroid hormone (PTH 1-84) ELISA kit (Immutopics Inc.).

The sufficient number of streptavidin coated eight well strips were prepared in a 96-wells plate holder to run PTH standards, controls and unknown samples, then 20 μL of standards, controls, and samples was pipetted in duplicate into the designated well. Following that, 50 μL of Working Antibody Solution consisting of equal volumes of mouse PTH 1-84 Biotinylated Antibody and mouse PTH 1-84 HRP Conjugated Antibody was added into each well. The plate was covered with a plate sealer and an aluminum foil again to block the light, then incubated for three hours at 25°C on a horizontal rotator set at 220 RPM. After uncovering the aluminum foil and the plate sealer, each well was washed by pipetting 350 μL of Washing Solution and completely removing the contents five times in a row. A hundred μL of ELISA HRP Substrate was pipetted into each of the wells, then the plate was incubated for 30 minutes at 25°C on a horizontal rotator set at 220 RPM, covered again with a plate sealer and an aluminum foil.

Before the addition of the ELISA Stop Solution, the plate sealer and aluminum foil were removed and the absorbance at 620 nm was read in a

microplate reader against the 0 pg/mL standard wells as a blank. After immediately adding 100 µL of ELISA Stop Solution into each well, the plate was mixed for 1 minute on a horizontal rotator. This time, the absorbance at 450 nm was read in the microplate reader against a reagent blank of 100 µL of HRP Substrate and 100 µL of ELISA Stop Solution. Two standard curves based on the two absorbance readings at 620 nm and 450 nm are required for the calculation of the sample results. The first reading taken at 620 nm is used for constructing the secondary standard curve which is applied only for sample results that fall between the value of the fifth and sixth standards. The second reading taken at 450 nm is for the primary standard curve which utilizes the absorbance values obtained with the first five standards. All sample results were within the analytical range of the primary standard curve, so the data read at 450 nm only was used for the calculation of serum PTH concentrations.

5. Quantification of serum calcium

Serum calcium (Ca) concentration was determined by ortho-cresolphthalein complexone (OCPC) method using AceChem Ca Kit (YD Diagnostics). This assay utilizes the reaction between calcium and ortho-cresolphthalein which forms a complex with a purple color that absorbs between 560 and 590 nm. The intensity of the color is proportional to the concentration of calcium in the sample.

To prepare the working reagent, equal volumes of Reagent A (assay buffer) and Reagent B (chromogenic reagent) were combined and vortexed lightly. Five μL of diluted standards and samples was pipetted in duplicate into wells of a clear bottom 96-well plate, then 250 μL of working reagent was added to each well and tapped lightly to mix. The plate was incubated for 5 minutes at 37°C in a water bath and the optical density was measured at 546 nm in the microplate reader. The concentration of Ca in the sample was calculated using the equation obtained from the standard curve.

6. RNA extraction and cDNA synthesis

After extracting total RNA and synthesizing cDNA from liver, kidney, and visceral adipose tissue (VAT), mRNA expression of vitamin D hydroxylase and VDR were analyzed by real-time PCR. Fifty to seventy mg of each tissue was homogenized in 1 mL of TRIzol reagent (Invitrogen) and incubated for five minutes at room temperature. Following the addition of 200 μL of chloroform, all samples were vortexed and incubated for 3 minutes, then centrifuged at 12,000 ×g for 15 minutes at 4°C. The separated supernatants were transferred to fresh tubes and 500 μL of isopropanol was pipetted to each tube. After 10 minutes of incubation at room temperature, all tubes were centrifuged at 12,000 ×g for 10 minutes at 4°C. The aqueous phase was removed by drying at room temperature and only RNA pellets were left in the tube. To wash the RNA pellet, 1 mL of 75% ethanol was added and vortexed, then centrifuged for 2 minutes at 4°C. Supernatants were decanted again and the left RNA pellets were dried in the air. The extracted total RNA solution was obtained after adding 20 μL of 0.2% diethylpyrocarbonate (DEPC)-treated water to dissolve the RNA pellet in each tube. The qualities of the RNA samples were examined by agarose gel electrophoresis using Gel Doc XR system (Bio-Rad Laboratories Inc.). The

concentration of RNA solutions was quantified by measuring absorbance at 260 nm and 280 nm using a spectrophotometer (DU530, BECKMAN).

Two μg portion of total RNA was reverse-transcribed into cDNA with PrimeScriptTM Ⅰ 1st strand cDNA synthesis kit (Takara Bio Inc.). The cDNA synthesis reaction was performed at 42°C for 50 minutes and stopped by denaturing at 95°C for 5 minutes using Applied Biosystems Thermal Cycler 2720 (Life technologies co.).

7. Real-time PCR for mRNA analysis

mRNA levels of 25-hydroxylases (CYP2R1, CYP27A1, CYP2J3) in liver and adipose tissue, 24-hydroxylase (CYP24) and 1α-hydroxylase (CYP27B1) in kidney, and VDR in adipose tissue were determined by real-time PCR. A total of 20 μL PCR reaction mixture containing the specific primers, reverse-transcribed cDNA, ROX reference dye, SYBR® Premix Ex Taq (Takara Bio Inc.) and autoclaved distilled water was prepared for each sample. The sequences of specific primers are shown in **Table 2**.

All PCR reactions were performed using StepOne™ Real-time PCR system (Applied Biosystems) under the following steps and conditions; initiation step at 95°C for 30 seconds, denaturation step at 95°C for 5 seconds, annealing and extension step at 60°C for 30 seconds up to 40 cycles, and melting curve analysis at 95°C for 15 seconds, 60°C for 1 minute, and 95°C for 15 seconds.

Table 2. Primer sequences used in real-time quantitative PCR

Gene	Function	Forward primer (5’ – 3’)	Reverse primer (5’ – 3’)
Cyp2r1	25-hydroxylation	TGGTGAGGTAAATGAGGCTTTC	TGCCAGTGCTCCAGTCTTC
Cyp27a1	25-hydroxylation	CCAAGGCAAGGTGGTAGAGA	CTTCATCGCACAAGGAGAGC
Cyp2j3	25-hydroxylation	ACCTCCTTTGCTCCTTCCAT	CAGCCACACCTATCCCTTCA
Cyp27b1	1-hydroxylation	GACGATGTTGGCTGTCTTCC	ATCTCTTCCCTTCGGCTTTG
Cyp24	24-hydroxylation	TCCCTGAGTAATGGGCTTTG	CACGGTAGGCTGCTGAGATT
Vdr	vitamin D receptor	ATGTCCAGTGAGGGGGTGTA	TGTCTGAGGAGCAACAGCAC
Gapdh	house-keeping gene	GGAGAAACCTGCCAAGTA	AAGAGTGGGAGTTGCTGTTG

8. Total protein extraction and western blot

Total protein was extracted from liver tissue and the expression of specific proteins was determined by western blot analysis. To extract total protein, 500 mg tissue was added to 500 μL of radioimmunoprecipitation assay (RIPA) buffer and homogenized on ice. The composition of RIPA is as follows; 50 μM Tris-Cl (pH 7.4), 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1mM EDTA, 1 mM PMSF, 1 mM Na_3VO_4, 1 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 10% glycerol, protease inhibitor cocktail 1 tablet/10 ml (Roche). The homogenates were incubated on ice for 30 minutes and centrifuged at 12,000 rpm for 20 minutes at 4°C. Supernatants were transferred to fresh tubes and concentrations of extracted total protein were determined by Bradford assay. BSA standard was diluted into 0.01, 0.02, 0.05, 0.1, 0.2, 0.5 and 1.0 mg/mL concentrations and 50X, 100X, and 200X diluted protein extracts were prepared. Ten μL of each standard and protein extract solution were pipetted into the appropriate well on 96-well plate, then 200 μL of 5X diluted Bradford assay reagent (Bio-Rad Laboratories Inc.) was added to each well. After incubating for 5 minutes at room temperature, the absorbance at 595 nm was measured. The most appropriate dilution concentration of protein extract was determined by using the stand-

ard curve, and the concentration of protein was calculated. Protein extracts were stored at −80°C before analysis.

Protein levels of 25-hydroxylase (CYP27A1) in liver were evaluated by western blot analysis. The 95:5 volume ratio of Laemmli sample buffer (Bio-Rad Laboratories Inc.)-β-mercaptoethanol solution and distilled water were added to protein extracts from liver and kidney to make appropriate concentration for each specific protein. Forty μg of protein samples were separated on a precast 10% SDS-PAGE gel and transferred to PVDF membrane (Bio-Rad Laboratories Inc.). Membranes were blocked overnight at 4°C in 5% skim milk solution (Tris buffer-saline solution with 0.1% Tween 20 and 5% skim milk), then incubated overnight at 4°C in 5% skim milk solution with goat anti-CYP27A1 (1:200 dilution, Santa Cruz Biotechnology Inc.) or with mouse anti-β-actin (1:10,000 dilution, Sigma-Aldrich Co.), and washed 4 times with TBST solution (Tris buffer-saline solution with 0.1% Tween 20). The blots were next applied in 5% skim milk solution with anti-goat IgG-HRP (1:8,500 dilution, Abcam plc.) or with anti-mouse IgG-HRP (1:20,000 dilution, Invitrogen) for 1 hour, then washed 4 times with TBST solution. Finally, the membranes were incubated in ECL solution (Animal Genetics Inc.) for 1 minute in the dark room and expressions of specific proteins were detected on X-ray film. Band density was analyzed using the

Quantity One (Bio-Rad Laboratories Inc.) software program with β-actin as loading control.

9. Statistical analysis

Statistical analysis was performed using SPSS statistical software (version 21.0; SPSS Inc.). To compare differences between control and obese groups, Mann-Whitney U-test or Student's t-test was used based on the results of Kolmogorov-Smirnov test for normal distribution. Correlations between two variables were assessed using the Pearson correlation test. *P*-value less than 0.05 was considered statistically significant.

IV. Results

1. Body weight, weight gain, white adipose tissue weight, and dietary intake

After 18-wk of feeding period, mice fed the high fat diet had significantly higher body weight (58% higher, $P < 0.001$) and white adipose tissue weight (90% higher, $P < 0.001$) compared with the mice fed the control diet **(Figure 3)**. Van Heek et al. (Van Heek et al., 1997) observed high fat diet (45% kcal fat diet) group became 25% heavier than control diet (10% kcal fat diet) group in C57BL/6 mice, and reported that obesity was induced in high fat diet group by feeding high fat diet contained 45% kcal as fat over a period of 51 days. Thus, we could speculate that obesity was successfully induced in high fat diet fed mice in this study and these mice could be considered as obese group.

Although daily food intake was 12% lower in the obese group, daily energy intake was 9% higher because of the higher percent of fat in the high fat diet. High fat diet contained 23% higher vitamin D and Ca than control diet (960 IU vitamin D and 5.0 g Ca per kg diet in control vs. 1180 IU vitamin D and 6.1 g Ca per kg diet in high fat), but daily intake of vitamin D and Ca were 6% higher in the obese group due to lower daily food intake in obese group **(Table 3)**.

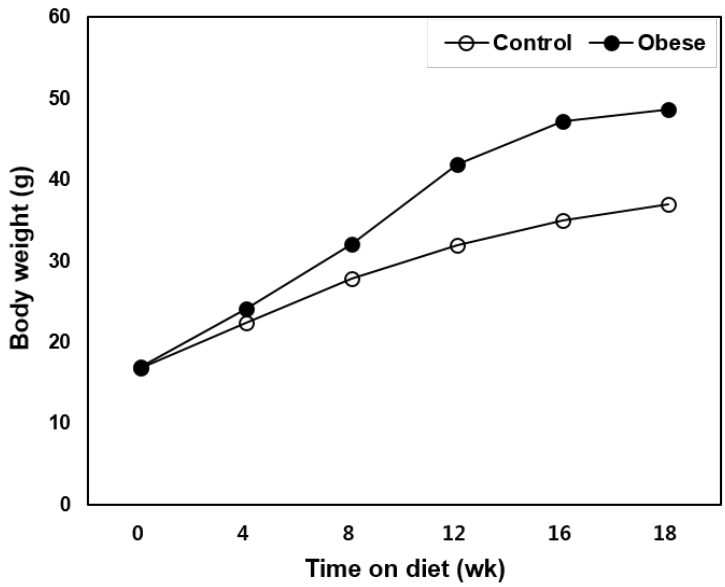

Figure 3. Body weight change of the mice fed control or high fat diets

Mice were fed control (10% energy as fat) or high fat (45% energy as fat) diets for 18-wk of feeding period. Values are expressed as means, n = 14 for each group.

Table 3. Body weight, weight gain, white adipose tissue weight, and dietary intake of the mice fed control or high fat diets[1]

	Control	Obese	*P*-value[2]
Body weight at 0 week, *g*	16.96 ± 0.31	16.77 ± 0.25	0.643
Body weight at 18 week, *g*	37.53 ± 0.76	49.24 ± 0.31	< 0.001
Body weight gain, *g*	20.57 ± 0.94	32.46 ± 0.28	< 0.001
White adipose tissue weight, *g*	3.94 ± 0.17	7.47 ± 0.16	< 0.001
Daily food intake, *g/d*	3.44 ± 0.04	3.02 ± 0.04	< 0.001
Daily vitamin D intake, *IU/d*	3.29 ± 0.04	3.56 ± 0.05	< 0.001
Daily calcium intake, *mg/d*	17.48 ± 0.22	18.51 ± 0.25	< 0.001
Daily energy intake, *kcal/d*	12.73 ± 0.16	13.89 ± 0.18	< 0.001

[1] Data are presented as means ± SEM, n = 14 for each group.

[2] Student's t-test was performed to determine the significant effect of obesity. A *P*-value of < 0.05 was considered statistically significant.

2. Serum concentrations of vitamin D metabolites and calcium

Serum concentrations of 25(OH)D and 1,25$(OH)_2$D were measured in order to investigate whether circulating levels of major vitamin D metabolites were influenced by obesity. There was no difference in serum 25(OH)D concentration between control group (27.63 ± 0.73 ng/mL) and obese group (30.30 ± 1.44 ng/mL) (**Figure 4A**), while serum 1,25$(OH)_2$D concentration was significantly higher ($P < 0.05$) in obese group (222.00 ± 40.56 pg/mL) than control group (107.12 ± 14.37 pg/mL) (**Figure 4B**). Serum Ca concentrations were not significantly different between control group (12.58 ± 0.31 mg/dL) and obese group (13.38 ± 0.57 mg/dL) **(Figure 4C)**.

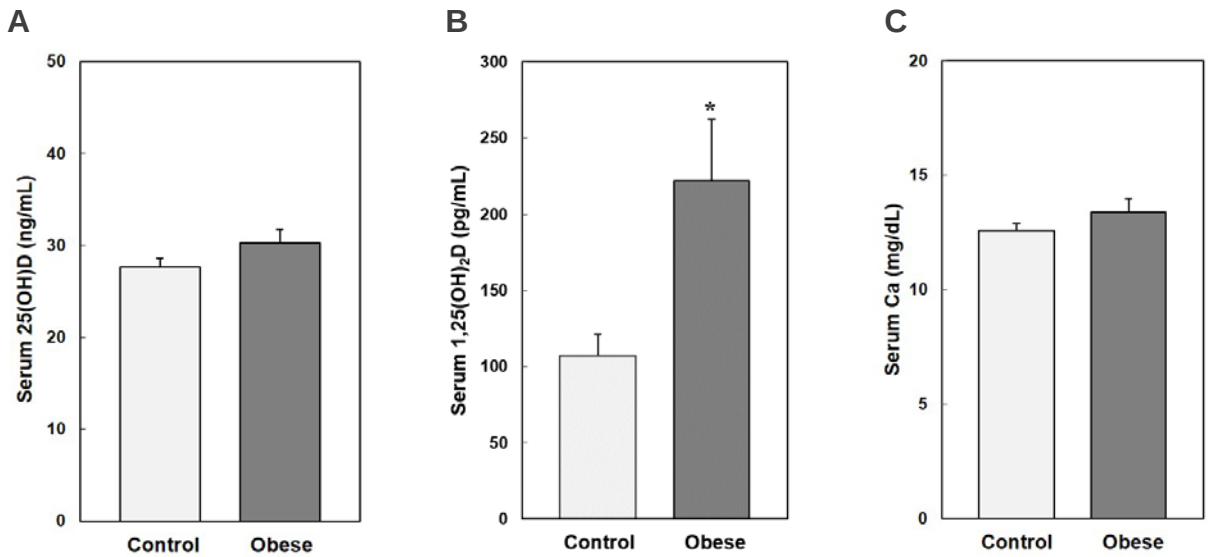

Figure 4. Serum concentrations of 25(OH)D (A), 1,25(OH)$_2$D (B), and calcium (C)

Serum vitamin D metabolites' levels were measured by radioimmunoassay and serum calcium levels were measured by ortho-cresolphthalein complexone method. Values are expressed as means ± SEM, n = 10 for serum 25(OH)D, n = 6 for serum 1,25(OH)$_2$D and n = 9 for serum calcium for each group. *Significantly different compared with the control by Student's t-test, $P < 0.05$.

3. Serum concentration of parathyroid hormone

To examine whether secondary hyperparathyroidism, which could influence the regulation of renal 1α-hydroxylase, occurred in obesity, serum concentration of PTH was analyzed. Obese group had significantly higher ($P < 0.05$) serum PTH concentration than control group (161.84 ± 22.12 pg/mL in obese vs. 100.66 ± 13.28 pg/mL in control) **(Figure 5)**, and serum concentration of PTH correlated positively with body weight ($r = 0.58$, $P < 0.05$).

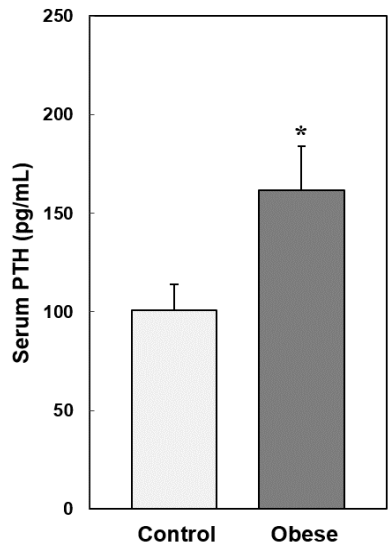

Figure 5. Serum concentration of PTH

Serum PTH levels were measured by two-site enzyme-linked immunosorbent assay. Values are expressed as means ± SEM, n = 6 for each group. *Significantly different compared with the control by Student's t-test, $P < 0.05$.

4. Expression of hepatic 25-hydroxylases

To evaluate whether the major contributors of the 25(OH)D synthesis were affected by obesity, expression of 25-hydroxylases in liver was determined. The mRNA levels of three hepatic 25-hydroxylases, *Cyp2r1*, *Cyp27a1* and *Cyp2j3*, were significantly lower (31% lower, $P < 0.001$; 30% lower, $P < 0.05$; 48% lower, $P < 0.05$, respectively) in obese group than control group (**Figure 6A**).

In order to find out whether differences in 25-hydroxylase mRNA level resulted in changes at the protein level, CYP27A1 protein expression was measured. However, the significant difference in the expression of *Cyp27a1* at mRNA level between control and obese groups was not sustained at the protein level as there was no significant difference in CYP27A1 protein levels (**Figure 6B**).

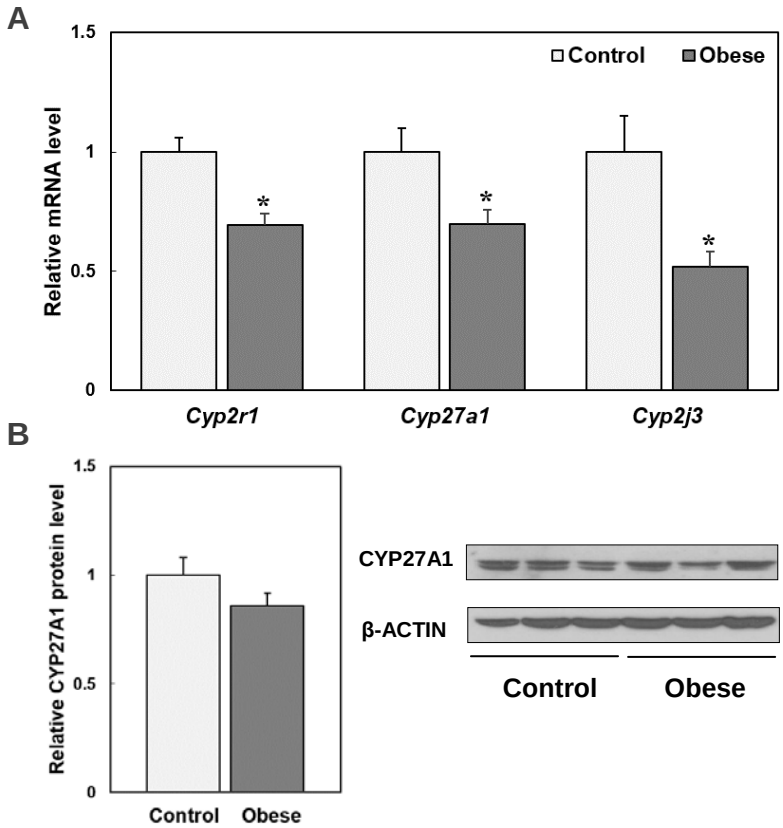

Figure 6. Hepatic expression of vitamin D 25-hydroxylases. mRNA levels of *Cyp2r1*, *Cyp27a1*, and *Cyp2j3* (A) and protein level of CYP27A1 (B) The mRNA levels were measured by quantitative RT-PCR. All levels were normalized to the levels of house-keeping gene, *Gapdh*, and expressed as relative values compared with the control (**A**). The protein expression level was analyzed by western blot and the quantification of the blot was done by densitometric analysis. CYP27A1 protein expression level was normalized to the protein expression level of β-actin and presented as relative values compared with the control (**B**). Values are expressed as means ± SEM, n = 10 for each group. *****Significantly different compared with the control by Student's t-test, $P < 0.05$.

5. Expression of renal 1α-hydroxylase and 24-hydroxylase

Serum 1,25$(OH)_2$D concentration is regulated by the balance between activation of 25(OH)D by renal 1α-hydroxylase and inactivation of 1,25$(OH)_2$D by renal 24-hydroxylase. As significantly higher serum 1,25$(OH)_2$D concentration was observed in obese group, we examined whether there were any changes in the expression of these two renal hydroxylases due to obesity. Renal *Cyp27b1* mRNA level was upregulated (517% higher, $P < 0.01$) and *Cyp24* mRNA level was downregulated (48% lower, $P < 0.05$) in obese group compared with control group (**Figure 7)**. *Cyp27b1* mRNA level correlated positively with serum 1,25$(OH)_2$D concentration ($r = 0.74$, $P < 0.01$) (**Figure 8A**), and *Cyp24* mRNA level showed negative correlation with serum 1,25$(OH)_2$D concentration ($r = -0.68$, $P < 0.01$) (**Figure 8B**).

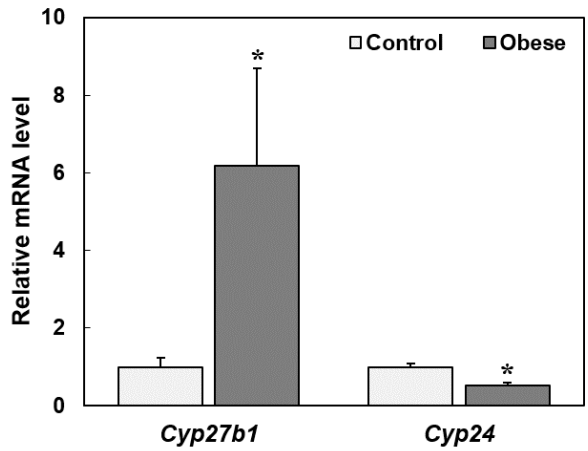

Figure 7. Renal expression of vitamin D 1α-hydroxylase and 24-hydroxylase

The mRNA levels were measured by quantitative RT-PCR. All levels were normalized to the levels of house-keeping gene, *Gapdh*, and expressed as relative values compared with the control. Values are expressed as means ± SEM, n = 10 for each group. *****Significantly different compared with the control either in renal 1α-hydroxylase expression by Mann-Whitney U-test or in renal 24-hydroxylase expression by Student's t-test, $P < 0.05$.

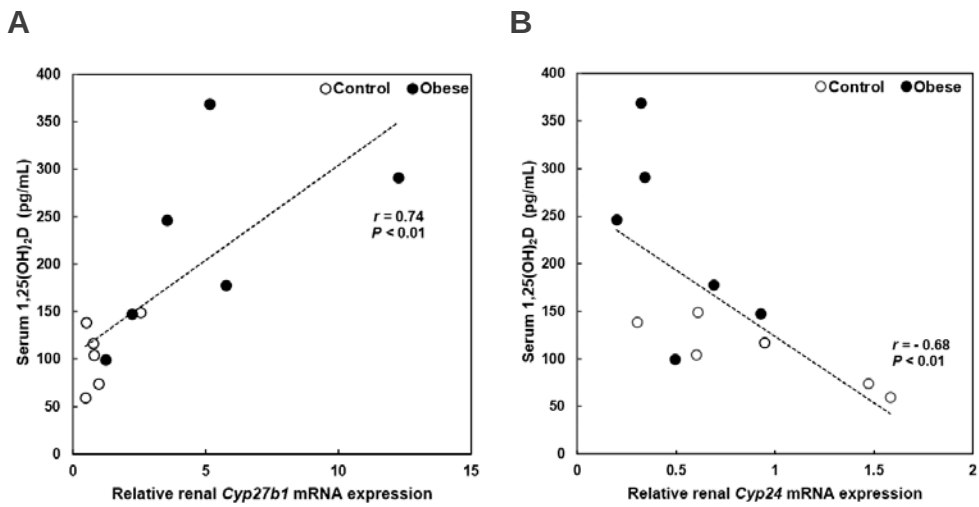

Figure 8. Relationship between serum 1,25(OH)$_2$D and renal *Cyp27b1* mRNA level (A) or renal *Cyp24* mRNA level (B)

Pearson correlation test was performed to determine the association between serum 1,25(OH)$_2$D and renal mRNA expressions of *Cyp27b1* (**A**) or *Cyp24* (**B**), and the coefficient of determination of each correlation is shown.

6. Expression of 25-hydroxylases and vitamin D receptor in visceral adipose tissue

To evaluate the existence of local vitamin D metabolism in adipose tissue, mRNA levels of vitamin D hydroxylases in VAT were measured. Obese group had significantly higher mRNA levels of 25-hydroxylases, *Cyp27a1* and *Cyp2j3* (68% higher, $P < 0.05$; 126% higher, $P < 0.01$ respectively), compared with control group (**Figure 9A**). However, 1α-hydroxylase (*Cyp27b1*) and 24-hydroxylase (*Cyp24*) were not detectable in VAT from mice in this study.

In order to determine whether expression of VDR in adipose tissue is influenced by obesity, we measured *Vdr* mRNA levels in VAT, and found that obese group had significantly higher *Vdr* mRNA level than control group ($P < 0.01$) (**Figure 9B**).

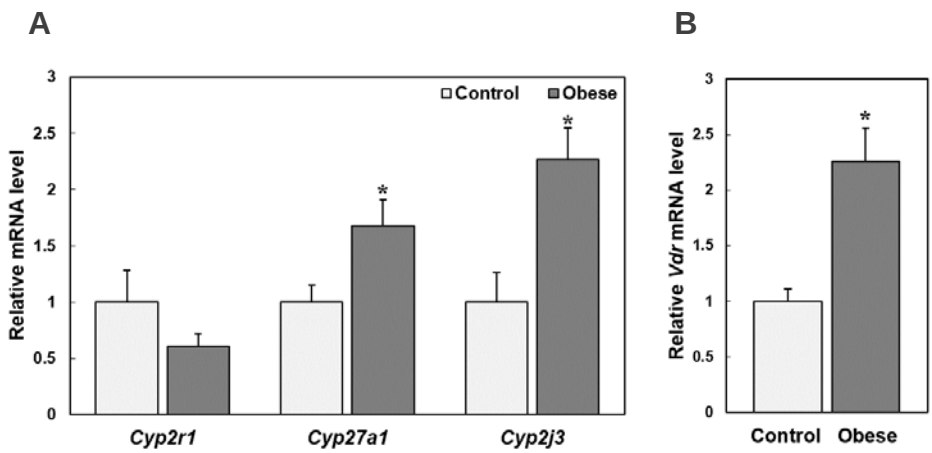

Figure 9. Expression of vitamin D 25-hydroxylases (A) and *Vdr* (B) in visceral adipose tissue

The mRNA levels were measured by quantitative RT-PCR. All levels were normalized to the levels of house-keeping gene, *Gapdh*, and expressed as relative values compared with the control. Values are expressed as means ± SEM, n = 5-6 for each group. *Significantly different compared with the control by Student's t-test, $P < 0.05$.

V. Discussion

In this study, we investigated the impact of obesity on vitamin D status and the possible underlying mechanism of altered vitamin D metabolism by examining the expressions of genes and protein related to vitamin D metabolism in high fat diet-induced obese mice. An abnormal regulation of serum $1,25(OH)_2D$ was observed in obese mice, which seemed to be associated with increased PTH level and explained by alterations in the expression of major vitamin D metabolizing enzymes, 1α-hydroxylase and 24-hydroxylase, in the kidney.

There was no difference in circulating 25(OH)D concentration between control and obese groups in this study. In human, however, obesity has often been associated with low status of vitamin D (Earthman et al., 2012). Among several possible explanations for vitamin D deficiency in obese people, inadequate sun exposure and suboptimal dietary vitamin D intake were often suggested (Earthman et al., 2012). The vitamin D content of two experimental diets in this study were 960 IU/kg in control diet and 1180 IU/kg in high fat diet, which were close to NRC requirement for vitamin D in rodents' diets, 1000 IU/kg (Subcommittee on Laboratory Animal Nutrition, 1995). Although high fat diet contained 23% more vitamin D per weight diet than the control diet, the actual intake of vitamin D was 6%

more in the obese group because of less food intake in high fat diet-fed mice. In a study which observed the significant difference in serum concentrations of 25(OH)D and 1,25(OH)D by dietary vitamin D supplementation in rats, 257% more vitamin D was fed in the vitamin D-supplemented diet group than the conventional diet group (Vieth et al., 2000). Therefore, 6% difference in dietary vitamin D intake between two groups in this study is not likely to have significant influence on serum vitamin D concentrations.

Although serum 25(OH)D concentration was not significantly different in obese group compared with control group, mRNA levels of hepatic 25-hydroxylases, *Cyp2r1*, *Cyp27a1*, and *Cyp2j3*, were significantly lower in obese group. Protein expression of 25-hydroxylase, CYP27A1, was similar between control and obese groups despite a difference in mRNA levels, which might explain no difference in serum 25(OH)D level between two groups. In a study which investigated the expression of enzymes involved in vitamin D metabolism in endometriosis and ovarian cancer, the observed difference in the *Cyp27a1* mRNA level in ovaries between control group and ovarian cancer group was not maintained at the protein level (Agic et al., 2007). There is a possibility that protein expression of other two 25-hydroxylases, CYP2R1 and CYP2J3, might be different. However, mitochondrial vitamin D 25-hydroxylase was shown to have 1.5 fold higher activity than microsomal vitamin D 25-hydroxylase in rat liver (Masumoto et

al., 1988), and we observed more abundant expression of mitochondrial 25-hydroxylase, *Cyp27a1*, than two microsomal 25-hydroxylases, *Cyp2r1* and *Cyp2j3*, in the liver. Therefore, even if there is a differential protein expression of other two 25-hydroxylases, it may not have a significant influence on the entire hepatic 25-hydroxylation of vitamin D.

In adipose tissue, expressions of 25-hydroxylases, *Cyp27a1* and *Cyp2j3*, were more pronounced in obese mice. These findings suggest a possibility of promoted 25-hydroxylation in adipose tissue from obese mice, resulting in higher local production of 25(OH)D in adipose tissue. According to the recently proposed volumetric dilutional model of vitamin D_3 between serum and adipose tissue, the synthesized or ingested vitamin D_3 would be distributed to the serum and adipose tissue in a simple bidirectional and diffusional equilibrium (Drincic et al., 2012). Further, it was suggested that similar mechanism with regard to 25(OH)D might exist between serum and adipose compartments (Piccolo et al., 2013). In obese mice in this study, more 25(OH)D could be synthesized from adipose tissue due to higher 25-hydroxylase expression and more adiposity with obesity, which might have an influence on the circulating level of 25(OH)D and could explain similar levels of serum 25(OH)D despite a lower expression of hepatic 25-hydroxylases observed in obese mice.

In this study, $1,25(OH)_2D$, an important vitamin D metabolite in circulation and whose level is tightly regulated in normal physiological condition, was significantly higher in obese group. Serum concentration of PTH was measured because PTH is the principal regulator of the renal $1,25(OH)_2D$ synthesis by stimulating 1α-hydroxylase. Higher concentration of PTH was observed in the obese mice, although there was no difference in serum Ca concentration between two groups. In addition, serum PTH level correlated positively with the body weight in this study. Bell et al. (Bell et al., 1985) observed higher serum PTH and $1,25(OH)_2D$, and lower serum 25(OH)D in obese subjects compared with the nonobese subjects despite a similar serum calcium and phosphorus levels when they were maintained for same dietary calcium and phosphorus intake for 2 days. They suggested altered vitamin D endocrine system due to secondary hyperparathyroidism, leading to increased renal production of $1,25(OH)_2D$, however, direct evidence or mechanism for increased $1,25(OH)_2D$ was not provided.

Mechanism of elevated $1,25(OH)_2D$ concentration with obesity was further investigated by looking at the mRNA expressions of renal *Cyp27b1* and *Cyp24*. Renal *Cyp27b1* mRNA expression was upregulated and *Cyp24* mRNA expression was downregulated in the obese mice, which is the first observation to our knowledge. Furthermore, serum $1,25(OH)_2D$ level showed positive correlation with renal *Cyp27b1* mRNA expression and neg-

ative correlation with renal *Cyp24* mRNA expression. When varied amount of dietary Ca intake was used to modulate $1,25(OH)_2D$ levels in rats, serum $1,25(OH)_2D$ levels had positive relationship with renal *Cyp27b1* mRNA levels and negative relationship with renal *Cyp24* mRNA levels (Anderson et al., 2004). This result clearly showed that renal mRNA levels of *Cyp27b1* and *Cyp24* are closely associated with serum $1,25(OH)_2D$ level. Thus, higher serum $1,25(OH)_2D$ level in obese mice in this study could be attributed to the alterations in the expression of renal 1α-hydroxylase and 24-hydroxylase. In the present study, difference in dietary Ca intake between control and obese groups was small and circulating Ca level was similar between two groups, but significant differences in renal mRNA expression of *Cyp27b1* and *Cyp24* between two groups were observed. PTH was reported to stimulate renal *Cyp27b1* expression (Brenza et al., 2000) and attenuate renal *Cyp24* expression (Zierold et al., 2000). The treatment of PTH in AOK-B50 cells, a porcine proximal tubule cell line with stably transfected opossum PTH receptor, and in HKC-8 cells, an SV40 transformed human kidney cell line, rapidly increased *Cyp27b1* transcript level (Brenza et al., 2000). *Cyp24* mRNA expression and activity were repressed by PTH in AOK-B50 cells (Zierold et al., 2000). The increases in PTH due to obesity in this study might be responsible for the higher *Cyp27b1* and lower *Cyp24* expression,

resulting in stimulation of renal 1,25$(OH)_2$D production and subsequent increase in serum 1,25$(OH)_2$D.

Available evidence by *in vivo* study in normal human subjects indicated that 1,25$(OH)_2$D inhibited the hepatic production of 25(OH)D (Bell et al., 1984), and it was reported that the reduction in serum 25(OH)D in obese human might be attributed to this feedback regulation by elevated 1,25$(OH)_2$D (Bell et al., 1985). However, these previous studies could not provide the direct mechanism for the feedback regulation of 25(OH)D production. In the present study, we observed that mRNA expressions of all three hepatic 25-hydroxylases were lower in obese mice and expression of each hydroxylase showed negative correlation with serum 1,25$(OH)_2$D, especially *Cyp2r1* mRNA expression showed significantly negative correlation ($r = -0.67$, $P < 0.05$) with serum 1,25$(OH)_2$D. This might indicate a feedback inhibition of hepatic 25-hydroxylation at mRNA level by the increased level of serum 1,25$(OH)_2$D with obesity.

The biologically active form of vitamin D, 1,25$(OH)_2$D, performs its functions by interacting with nuclear VDR. We observed that *Vdr* mRNA was expressed in VAT of both mice groups, and obese group had higher *Vdr* expression compared with control group. It was suggested that 1,25$(OH)_2$D exerted nonclassic actions mediated by nVDR on adipocyte such as inhibiting UCP2 expression and thereby suppressing energy expenditure. The sup-

pression of 1,25$(OH)_2$D by increasing dietary calcium not only increased UCP2 expression but also stimulated lipolysis and inhibited lipogenesis, resulting in reduced adiposity (Shi et al., 2001, Zemel et al., 2000). As both serum 1,25$(OH)_2$D level and *Vdr* mRNA expression in adipose tissue were higher in obese mice, there is a possibility that increased access of 1,25$(OH)_2$D to adipose tissue and elevated interaction with nVDR would further stimulate adiposity.

In conclusion, high fat diet-induced obesity influenced the expression of vitamin D metabolizing enzymes, which could provide possible mechanisms of altered vitamin D metabolism in obesity. Higher concentration of serum PTH in obese mice might have contributed to the abnormal regulation of serum 1,25$(OH)_2$D concentration by stimulating 1α-hydroxylase expression and attenuating 24-hydroxylase expression in kidney. The increase in serum 1,25$(OH)_2$D could have potentially inhibited hepatic synthesis of 25(OH)D at mRNA level. Further, higher expression of 25-hydroxylases and VDR in adipose tissue might suggest the possibility of enhanced local 25(OH)D production and nonclassic functions by the interaction between 1,25$(OH)_2$D and VDR in adipose tissue.

VI. Summary

In this study, we investigated the vitamin D status and the expression of enzymes involved in vitamin D metabolism using high fat diet-induced obese mice in order to find out the possible underlying mechanism responsible for obesity related changes in vitamin D status. Mice were fed control diet containing 10% energy fat (control group) or high fat diet with 45% energy fat (obese group) for 18 weeks, then serum concentrations of vitamin D metabolites, calcium, PTH and the expression of enzymes involved in vitamin D metabolism were analyzed. The results obtained in the present study can be summarized as follows:

1) Obese group had significantly higher body weight and white adipose tissue weight than control group. Daily intake of vitamin D and Ca were 6% higher in the obese group.
2) No difference was observed in serum 25(OH)D concentration between control group and obese group. But, obese group had significantly lower mRNA levels of three hepatic 25-hydroxylases, *Cyp2r1*, *Cyp27a1* and *Cyp2j3* compared with the control group. There was no significant difference in CYP27A1 protein levels between two groups.
3) While serum Ca concentration was not significantly different between two groups, serum concentrations of 1,25$(OH)_2$D and PTH were signifi-

cantly higher in obese group. Serum concentration of PTH showed positive correlation with body weight.

4) In obese group, renal 1α-hydroxylase (*Cyp27b1*) mRNA level was upregulated and 24-hydroxylase (*Cyp24*) mRNA level was downregulated compared with control group. *Cyp27b1* mRNA level correlated positively with serum $1,25(OH)_2D$ concentration and *Cyp24* mRNA level showed negative correlation with serum $1,25(OH)_2D$ concentration.
5) Obese group had significantly higher mRNA levels of *Vdr* and 25-hydroxylases, *Cyp27a1* and *Cyp2j3*, than control group.

The results from this study suggested that high fat diet-induced obesity influenced the expression of vitamin D metabolizing enzymes, which might partly explain possible mechanisms of altered vitamin D metabolism in obesity. Higher concentration of serum PTH due to obesity might have induced abnormal regulation of serum $1,25(OH)_2D$ concentration by stimulating renal 1α-hydroxylase expression and attenuating renal 24-hydroxylase expression. Hepatic 25-hydroxylation might have been inhibited by increased serum $1,25(OH)_2D$ concentration at mRNA level. Higher mRNA expression of VDR in adipose tissue might suggest the possibility of enhanced nonclassic functions by the interaction between $1,25(OH)_2D$ and VDR in adipose tissue.

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국문초록

고지방 식이로 유도한 비만이 마우스의 비타민 D 대사 관련 효소 발현에 미치는 영향

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박 정 민

비만인 사람에게서 비타민 D 결핍증 또는 부족증이 흔하게 나타나고 그 판단 지표인 혈청 25(OH)D 농도가 낮음이 보고되고 있다. 비만인에서의 혈청 25(OH)D 농도 저하와 관련하여, 외부 활동 감소로 인한 햇빛으로부터의 비타민 D 합성 부족, 간에서의 25(OH)D 생성 저해 등의 설명이 제시되어 왔으나, 아직 정확한 기전은 밝혀지지 않았다. 본 연구에서는 고지방 식이로 유도한 비만 마우스에서 비타민 D 대사물질 수준과 관련 효소 발현을 측정하여, 비만으로 인해 비타민 D 대사에 이상이 일어나는지를 확인하고 그 잠재적인 기전을 파악하고자 하였다. 4 주령의 C57BL/6 마우스를 두 군에 배정하여 대조군은 총 식이 열량의 10%를 지방으로 공급하는 일반식이를, 비만군은 총 식이 열량의 45%를 지방으로 공급하는 고지방식이를

18 주간 자유 급여하였다. 혈청 25(OH)D 농도는 군 간 차이가 없었으나, 혈청 1,25$(OH)_2$D 농도는 비만군에서 유의적으로 높았다. 간 조직에서 25-hydroxylase 의 mRNA 발현은 비만군에서 세 종류의 25-hydroxylases 모두 유의적으로 낮았다. 하지만 CYP27A1 의 단백질 발현은 군 간에 차이가 없었다. 비만군의 신장 조직에서 1α-hydroxylase (*Cyp27b1*) 의 mRNA 발현은 유의적으로 높았고, 24-hydroxylase (*Cyp24*) 의 mRNA 발현은 유의적으로 낮았다. 혈청 1,25$(OH)_2$D 농도는 신장의 *Cyp27b1* mRNA 발현 수준과 양의 상관관계를, *Cyp24* mRNA 발현 수준과는 음의 상관관계를 보였다. 혈청 칼슘 농도는 군 간 차이가 없었으나, 혈청 PTH 농도는 비만 마우스에서 더 높게 나타났다. 또한, 혈청 PTH 의 농도는 체중과 유의적인 양의 상관관계를 보였다. 백색지방 조직에서는 25-hydroxylase 인 *Cyp27a1* 와 *Cyp2j3* 의 mRNA 수준과 vitamin D receptor (*Vdr*)의 mRNA 수준이 비만군에서 더 높았다. 결론적으로, 비타민 D 대사 관련 효소의 발현은 고지방 식이로 유도한 비만에 의해 영향을 받았으며, 이는 비만에서의 비타민 D 대사 변화 기전을 일부 설명할 수 있다. 비만으로 인한 PTH 증가가 혈청 1,25$(OH)_2$D 의 높은 농도에 기여한 것으로 보이며, 이는 비만 마우스에서 관찰된 신장 1α-hydroxylase 의 발현 증가와 신장 24-hydroxylase 의 발현 감소에 의한 것으로 사료된다. 비만으로 인해 비정상적으로 높아진 혈청

1,25(OH)$_2$D 의 농도가 간에서의 25-hydroxylase 의 발현을 mRNA 수준에서 낮게 하는 데에 기여했을 가능성이 있다. 비만 마우스의 내장지방 조직에서 25-hydroxylase mRNA 와 *Vdr* mRNA 발현의 증가는 지방 조직 내 국부적인 25(OH)D 생성 및 1,25(OH)$_2$D 와 VDR 결합에 의한 nonclassic functions 의 증가 가능성을 제시한다.

주요어: 비만, 25-hydroxyvitamin D, 1,25-dihydroxyvitamin D, 간의 25-hydroxylase, 신장의 1α-hydroxylase, 신장의 24-hydroxylase, 고지방 식이를 섭취한 마우스

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